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ORIGINAL ARTICLE

Chemical composition of horse-chestnut (*Aesculus*) leaves and their susceptibility to chestnut leaf miner *Cameraria ohridella* Deschka & Dimić

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Abstract For over 20 years, trees of *Aesculus* spp. have been attacked by the larvae of *Cameraria ohridella*, which causes damage to the leaves. It has been observed that members of the genus *Aesculus* are characterized by diverse susceptibility to *C. ohridella*. Four specimens of the *Aesculus* genus which differ in susceptibility to this leaf miner—*Aesculus turbinata* (susceptible), *Aesculus* × *neglecta* (resistant) and two specimens of *Aesculus hippocastanum* (relatively susceptible and relatively resistant)—were examined. The levels of substances which may function as attractants (chloroplast pigments, anthocyanins), deterrents or repellents (flavonols, phenols), or a source of nutrients (free α-amino acids and carbohydrates) were determined in leaves of these four trees during two growing seasons. The results showed that the more pest-susceptible *A. turbinata* had, in both growing seasons, significantly higher levels of leaf carbohydrates and anthocyanins than the resistant *Ae.* × *neglecta*. Thus, anthocyanins and carbohydrates may be the traits which affect oviposition preference and favor the feeding of *C. ohridella* in the susceptible *Ae. turbinata*. The relatively susceptible specimen of *Ae. hippocastanum* contained slightly higher carbohydrate and anthocyanin levels than the relatively resistant one, but only in one growing season. Therefore, it does not explain the causes of their different susceptibility to the pest. The concentration of phenolics in the susceptible *Ae. turbinata* tree and the relatively susceptible *Ae. hippocastanum* individual was higher than in the resistant *Ae.* × *neglecta* and relatively resistant *Ae. hippocastanum*, respectively. This may suggest that leaf phenolic composition, but not overall concentration, is responsible for different susceptibility of examined trees of *Aesculus* spp. to the horse-chestnut leaf miner. The present results also demonstrate that the determined chemical compounds do not constitute a complete description of the biochemical relationships between *C. ohridella* and the examined horse-chestnut trees.

Keywords Horse-chestnut · *Cameraria ohridella* · Phenolics · Amino acids · Carbohydrates · Plant pigments

Introduction

For over 20 years, a threat to the esthetic values of horse-chestnut has been posed by the inconspicuous moth horse-chestnut leaf miner (*Cameraria ohridella* Deschka & Dimić, Lepidoptera: *Gracillariidae*), which was first recorded in Macedonia in the 1970s and rapidly spread throughout Central and Western Europe (Valade et al. 2009). The economic cost arising from the protection of *Aesculus* trees against this pest is substantial. For example, in Germany current costs of five major urban centers range from 10,020,000 to 33,800,000 € annually, but if trees in green spaces died, replacement costs would run up to 10.7 billion € (Reinhardt et al. 2003).

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The species of the genus *Aesculus* are characterized by diverse susceptibility to *C. ohridella*. The main host of this pest is the white horse-chestnut (*Aesculus hippocastanum* L.), which is native to the Balkans and since the end of the sixteenth century has been introduced throughout Europe as an attractive ornamental tree (Prada et al. 2011). However, considerable variation in susceptibility to *C. ohridella* has been observed between *Ae. hippocastanum* individual trees (Straw and Tilbury 2006; Irzykowska et al. 2013). Also susceptible to *C. ohridella* is the Japanese horse-chestnut (*Aesculus turbinata* Blume). Resistant species include *Aesculus indica* (Wall. ex Camb.) Hook, *Aesculus californica* (Spach) Nutt., *Aesculus parviflora* Walter, *Aesculus assamica* Griff., and *Aesculus wilsoni* Rehder, which are of Asian and/or of North American origin (Straw and Tilbury 2006; D'Costa et al. 2013). Likewise, some species of North American origin (*Aesculus sylvatica* Bartram, *Aesculus pavia* L., *Aesculus flava* Aiton, *Aesculus glabra* Wild) are colonized by females that deposit eggs on the leaf surface but most larvae die during development (Kenis et al. 2003; Ferracini et al. 2010). Also resistant to *C. ohridella* is the hybrid of European *Aesculus hippocastanum* L. and American *Aesculus pavia* L. named red horse-chestnut (*Aesculus* × *caenea* Hayne), a popular tree in Europe (Irzykowska et al. 2013).

Resistance or susceptibility to pests is the result of plant–pest interaction. There are three mechanisms of resistance to herbivores, which are based on the plant properties. Antixenosis includes all plant traits which prevent oviposition and feeding and makes the plant an unsuitable host. Antibiosis involves harmful effects of plant on insect and tolerance that is the ability of plant to withstand or repair damage caused by the herbivore. The essential aspect of the relationships between host plants and insects is the chemical composition of leaves, which is decisive for the occurrence of interactions that facilitate or hamper feeding (Mithöfer and Boland 2012).

Plant–pest communication can take place through visual stimuli (Lev-Yadun and Gould 2009; Kocíková et al. 2012). The green leaf blades of host plants are willingly attacked by herbivores whose caterpillars feed on their tissues (Karageorgou and Manetas 2006). Chloroplast pigments are necessary for proper function of photosynthesis and are indirectly responsible for the synthesis of carbohydrates, which are a valuable nutrient for phytophagous insects (Jonas and Joern 2013). Female horse-chestnut leaf miners also react to the appearance of leaves and deposit their eggs on leaves with a large proportion of green area, which are not already occupied by mines, because such leaves have an abundance of food for larval growth and development (Johne et al. 2006a). The presence of anthocyanin pigments in leaves may also be a visual signal to insects (Archetti and Brown 2004). It was observed that red cabbage having a higher anthocyanin concentration than green cabbage was more attractive for caterpillars of lepidopteran pests (Coelho 2004). On the other hand, red coloration of leaves may also act as a warning signal for plant-eating insects, indicating that plants are well defended or have poor nutritional quality (Lev-Yadun and Gould 2009; Karageorgou and Manetas 2006; Maskato et al. 2014). High anthocyanin level may provide a visual cue to insect herbivores about a relatively high level of leaf total phenolic compounds which inhibit feeding of larvae and lead to their death (Karageorgou et al. 2008; Bouzouina et al. 2012; Oszmiański et al. 2014). In addition, feeding of herbivorous insect may induce accumulation of phenolics in leaves of host plants (Bernards and Båstrup-Spohr 2008). Chemical compounds that may act as defensive agents as well as alimentary deterrents are flavonols, which inhibit the growth of larvae, causing an extension of the larval period and increased larval mortality (Adeyemi et al. 2010; Martemyanov et al. 2012).

The availability of high quality and quantity of nutritional compounds (proteins, carbohydrates) is also an essential factor in the feeding of many herbivorous insects (Zhang et al. 2011). Good nutrition is necessary for the longevity of oviposition period, lifetime fecundity and oviposition rate in Lepidoptera (Cahenzli and Erhardt 2013; Marchioro and Foerster 2013). Plant amino acids are a key source of nitrogen, which is a significant factor in egg production (Cahenzli and Erhardt 2013). Carbohydrates, which are also an important component in the diet of many Lepidoptera, cover energy requirements and provide carbon skeletons for somatic development, fitness and reproductive output (Glendinning et al. 2007; Marchioro and Foerster 2013). High activity of amylase, maltase and saccharase which was found in the midgut of *C. ohridella* suggests that starch, maltose and saccharose are primary carbohydrate components of their diet (Stygar et al. 2010).

The aim of the study was to examine the content of chemical compounds which may act as attractants (chloroplast pigments, anthocyanins), defensive substances (anthocyanins, phenols and flavonols) and nutrients (free α-amino acids and carbohydrates) in four *Aesculus* trees differing in susceptibility to *C. ohridella.* The research was focused on Japanese horse-chestnut (*Aesculus turbinata*), which is susceptible to this pest, resistant painted buckeye horse-chestnut (*Aesculus* × *neglecta* var. *lanceolata Sargent*, also known as *Aesculus sylvatica* Bartram), and two white horse-chestnut (*Aesculus hippocastanum*) trees that are growing close to each other and are not equally susceptible to this pest. One of them was colonized by the pest earlier, and the leaves are damaged to a greater extent than on the other tree.

These studies were undertaken in order to clarify the differences in resistance of the examined horse-chestnut trees to *C. ohridella* that we have observed for several years. According to the literature data, we assume that

horse-chestnut trees not infested or those on which the leaf miner does not continue feeding contain compounds acting harmfully on the pest population and do not contain nutritional compounds for this pest (Johne et al. 2006b; Kropczyńska-Linkiewicz et al. 2008). On the other hand, trees inhabited by *C. ohridella* are likely to contain substances working as attractants that make it easier for the insect to find the host plant and may have adequate quantity and quality of nutritional compounds (Johne et al. 2006a; Kropczyńska-Linkiewicz et al. 2008; Svatos et al. 2009; Marchioro and Foerster 2013). The differences in the chemical composition of the leaves of these *Aesculus* trees will allow the identification of substances which may be important in the interaction between *C. ohridella* and these trees. The obtained results can be used for further phytochemical studies on larger populations of horse-chestnut species.

Materials and methods

Weather data

Data on weather conditions in the successive years of study were obtained from the website of Poznań University of Life Sciences (http://www.au.poznan.pl/wogr/hobo/index.html). The measurements were performed by the meteorological Onset HOBO Weather Station installed in the Experimental Station of Poznań University of Life Sciences. Results are presented as means for each month in the period from March to October.

Plant material and sample collection

Four trees of the genus *Aesculus* were included in this study: two *Aesculus hippocastanum* trees growing at the campus of Poznań University of Life Sciences, one tree of *Ae. turbinata* and one of *Ae.* × *neglecta*, both located in the Botanical Garden of the Adam Mickiewicz University. Fully developed compound leaves were randomly chosen from the same location within the tree crown (similarly lighted place at the same height of each tree crown) every 14 days, at the same time of day (between 8 and 9 am) from May to September 2011 and 2012. Collected material was put into plastic bags and transferred to the laboratory within 30–60 min. Sample collection and laboratory assessment were carried out by two persons.

Estimation of damage of leaves by horse-chestnut leaf miner

The degree of leaf damage was evaluated visually by the same person by estimating the size of leaf blade occupied by mines in relation to the total leaf area and was expressed in percentages. Each time the assessment was performed on 15 compound leaves from each tree. After assessment the leaves were used for chemical analyses.

Chemical analyses

The content of chloroplast pigments in leaves was determined immediately after transfer to the laboratory. Plant material for the determination of other compounds was frozen in liquid nitrogen and stored at −20 °C until analysis. Only green parts of leaves (not occupied by mines) were taken for all evaluations. Chemical analyses were done during the entire growing season in the resistant *Ae.* × *neglecta* tree and the relatively resistant *Ae. hippocastanum* individual in both 2011 and 2012 and in the susceptible *Ae. turbinata* tree in 2012. However, on the sampling dates in which more than 60% of the leaf blade was occupied by mines chemical analyses were not carried out (*Ae. turbinata* tree from 14 July 2011, relatively susceptible *Ae. hippocastanum* tree from 14 July 2011 and 13 August 2012).

Each parameter measured for each tree, date and year was estimated in five independent replications. Each replication was a sample of plant material which derived from the middle leaflet of different compound leaves collected from the same tree.

Chloroplast pigments

Total chlorophyll and carotenoids content was estimated according to the method of Hiscox and Israelstam (1979). Leaf samples (100 mg) were cut into pieces and pigments were extracted at 65 °C using 5 cm^3 of dimethyl sulfoxide (DMSO). Optical density of the extract was measured at 480, 649 and 663 nm. The content of total chlorophyll and carotenoids was calculated following the modified Arnon equations (Wellburn 1994) and was expressed in milligrams per gram of fresh weight (mg g^{-1} FW).

Anthocyanins

Plant material (500 mg) was homogenized with 3 cm^3 of 0.5 N HCl, and centrifuged at 6000*g* for 10 min. Absorbance of the supernatant was measured at 530 nm (Wang et al. 2000). Anthocyanin content in leaf tissue was calculated using a calibration curve of cyanine chloride and was expressed in micrograms per gram of fresh weight (μg g^{-1} FW).

Total phenolic compounds

Leaf samples (250 mg) were cut into small pieces and extracted at 60 °C first with 5 cm^3 of 96% ethanol for

3 min and then with 5 cm^3 of 65% ethanol for 5 min. Each extract was poured into a test tube and it was filled up to the final volume of 10 cm^3. To 0.5 cm^3 of extract 0.1 cm^3 of Folin-Denis reagent was added and exactly 3 min later 1 cm^3 of 10% Na_2CO_3. After 20 min the absorbance was measured at 660 nm (Swain and Hillis 1959). The level of phenolics was calculated from the standard curve for coumaric acid and expressed in micrograms per gram of fresh weight (μg g^{-1} FW).

Flavonols

Leaf samples (500 mg) were cut into small pieces and homogenized with 5 cm^3 of methanol, HCl and distilled water (90:1:1, v/v/v). The solution was stirred and heated (60 °C) for 10 min, cooled at room temperature for 15 min and centrifuged at 6000*g* for 30 min (Day 1993). The absorbance of the supernatant was measured at 254 nm with a UV/visible spectrophotometer (Jasco V-530 UV–VIS Spectrophotometer). Flavonol content in leaf tissue was calculated using the calibration curves of quercetin (Stefova et al. 2001) and was expressed in micrograms per gram of fresh weight (μg g^{-1} FW).

Free α-amino acids

The ninhydrin method was applied to estimate the level of amino acids (Baily 1962). Leaf samples (250 mg) were cut into small pieces and homogenized with 3 cm^3 of ethanol. The homogenate was centrifuged at 6000*g* for 30 min. 0.1 cm^3 of the supernatant was mixed with 0.9 cm^3 of 0.4 M acetic acid buffer, pH 5 and 1 cm^3 of ninhydric reagent (2 g of ninhydrin + 50 cm^3 of 50% ethanol). The mixture was incubated at 100 °C for 15 min and then cooled. The absorbance of samples was determined at 570 nm. Amino acid content in leaf tissue was calculated using the calibration curves of alanine and was expressed in micrograms per gram of fresh weight (μg g^{-1} FW).

Total soluble carbohydrates

The anthrone colorimetric method was applied to measure carbohydrate content (Luo and Huang 2011). Leaf samples (250 mg) were cut into small pieces and extracted for 30 min at 60 °C using 4 cm^3 of 80% ethanol. 0.5 cm^3 of supernatant was mixed with 1 cm^3 of anthrone reagent (25 cm^3 H_2SO_4 + 5 mg anthrone reagent). After 15 min the absorbance of the colored reaction product was read at 620 nm. The amount of carbohydrates was calculated using a standard curve for glucose and was expressed in milligrams per gram of fresh weight (mg g^{-1} FW).

Statistical analysis

For evaluation of statistical differences between the analyzed individual trees of two species (*Ae. turbinata* vs. *Ae.* × *neglecta*) and two trees of the same species (*Ae. hippocastanum*) we used analysis of variance (ANOVA) with repeated measurements ($n = 5$), where the dependent variables were the data of successive sampling dates and independent variables were individual trees. All statistical analyses were conducted for the same number of sampling dates. The significance level was set at $\alpha = 0.05$. The differences were considered as statistically significant when p values were less than or equal to α. All numerical analyses were performed using the STATISTICA 10 package.

Results

Weather conditions

The weather conditions were slightly different in 2011 and 2012. The average precipitation was higher in 2012, mainly due to higher precipitation in the period from May to August (Table 1). The average humidity was at similar levels for most months in 2011 and 2012, except for June, when it was much lower in 2011 than in 2012. The

Table 1 Weather conditions from March to October (monthly means) during 2011 and 2012

Month	Temperature (°C)		Insolation (h)		Precipitation (mm)		Humidity (%)	
	2011	2012	2011	2012	2011	2012	2011	2012
March	3.7	6.0	153.2	140.0	16.2	11.2	73.9	76.3
April	14.6	10.9	173.7	196.2	7.2	16.4	63.7	68.7
May	12.0	15.6	241.3	241.9	9.4	47.6	62.4	63.9
June	18.7	16.2	226.4	161.3	55.0	94.4	65.4	75.6
July	16.7	19.3	86.0	199.0	146.4	126.2	80.4	76.3
August	18.8	18.7	201.7	179.8	17.4	33.4	75.2	75.6
September	15.1	14.4	169.3	143.7	30.0	20.6	78.1	78.3
October	8.8	10.0	85.6	81.2	20.6	18.0	86.1	84.0

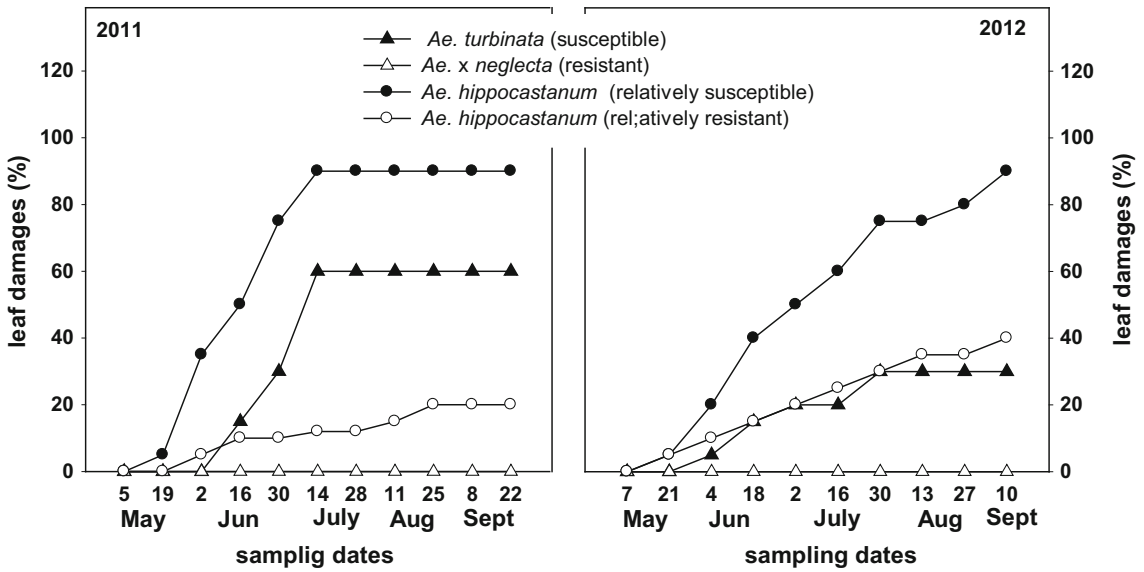

Fig. 1 The damage of leaf blades in Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in years 2011 and 2012

insolation measured on the basis of the number of sunny hours in 2011 was higher than in 2012 in five months (March, June, August, September and October), but in May it was similar in these two years. The average air temperature was lower in March and July, and higher in June, in 2011 in comparison to the same months in 2012, being at similar levels in the other months in both years.

Damage of leaves by the horse-chestnut leaf miner

In both years, greater damage of leaves was found in the relatively susceptible individual of *Ae. hippocastanum*. Similarly, the susceptible *Ae. turbinata* was characterized by greater damage than the resistant *Ae.* × *neglecta* (Fig. 1). Moreover, in the relatively susceptible *Ae. hippocastanum* tree and susceptible *Ae. turbinata* earlier and greater damage of leaf blades was found in 2011 than in 2012. This effect was probably caused by 2-week-earlier start of feeding by the pest in 2011 than in 2012. Already in mid-July 2011, almost 90% of the leaf blade area was covered with mines in the relatively susceptible tree of *Ae. hippocastanum*, and 60% in *Ae. turbinata.* In 2012 at the beginning of September 90% damage of the leaf blades was observed in the relatively susceptible white horse-chestnut tree and only 30% in *Ae. turbinata.* The leaf blades of resistant *Ae.* × *neglecta* were inhabited by single females but symptoms of feeding were never observed.

Results of chemical analyses

Statistically significant differences in the level of all estimated compounds throughout both years of experiments were observed between resistant *Ae.* × *neglecta* and susceptible *Ae. turbinata* (Table 2). In leaves of *Ae.* × *neglecta* higher levels of chlorophyll, carotenoids and α-amino acids were observed, while in *Ae. turbinata* there were higher levels of anthocyanins, phenols, flavonols and carbohydrates. Moreover, there were significant differences in time-dependent changes in the level of almost all examined compounds in these species, with the exception of chlorophyll in 2011 and carbohydrate and amino acids in 2012. In the two white horse-chestnut trees statistically significant differences were found only in the level of chlorophyll, anthocyanins, phenolics and flavonols, which were higher in the tree relatively susceptible to this pest (Table 3). The changes in the levels of chlorophyll, carotenoids and anthocyanins in these trees proceeded differently in time, in 2011. However, in 2012 different time-dependent changes were revealed in the level of almost all examined compounds with the exception of carotenoids and anthocyanins. The contents of particular compounds in the leaves of examined trees on successive sampling dates in 2011 and 2012 are shown in Figs. 2, 3, 4, 5, 6, 7, and 8.

Both in 2011 and 2012, the pest-resistant *Ae.* × *neglecta* was characterized by significantly higher total chlorophyll content in the leaf blades compared to susceptible Japanese horse-chestnut (Fig. 2). Likewise, in the leaves of the relatively resistant *Ae. hippocastanum* total chlorophyll content in both 2011 and 2012 was significantly higher than in the relatively susceptible one. However, these differences occurred only from mid-May to mid-June (Fig. 2).

Throughout the 2011 growing season the carotenoid content in the leaf blades of *Ae.* × *neglecta* was

Table 2 ANOVA results for the content of estimated compounds in the leaves of painted buckeye (*Aesculus* × *neglecta*) and Japanese horse-chestnut (*Aesculus turbinata*) in years 2011 and 2012

2011	*df*	Chlorophyll		Carotenoids		Anthocyanins		Phenolics		Flavonols		Carbohydrates		*df*	α-Aminoacids	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*		*F*	*p*
Individual	1	89.289	0.000	112.652	0.000	664.245	0.000	448.511	0.000	15.509	0.004	110.445	0.000	1	216.266	0.000
Sampling dates	4	4.079	0.009	4.167	0.008	3.620	0.015	13.646	0.000	12.362	0.000	5.903	0.001	3	44.016	0.000
Sampling dates × individuals	4	0.270	0.895	4.196	0.008	10.090	0.000	14.185	0.000	3.647	0.015	6.393	0.001	3	5.846	0.004
2012	*df*	Chlorophyll		Carotenoids		Anthocyanins		Phenols		Flavonols		Carbohydrates		*df*	α-Aminoacids	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*		*F*	*p*
Individual	1	53.4555	0.000	28.918	0.000	361.4990	0.000	475.251	0.000	137.4370	0.000	69.696	0.000	1	3102.573	0.000
Sampling dates	4	18.5160	0.000	81.523	0.000	66.5520	0.000	21.749	0.000	17.5060	0.000	6.609	0.001	3	17.665	0.000
Sampling dates × individuals	4	6.3398	0.001	7.737	0.000	10.7770	0.000	17.151	0.000	8.2190	0.000	2.519	0.061	3	2.281	0.105

Table 3 ANOVA results for the content of estimated compounds in the leaves of two trees of white horse-chestnut (*Aesculus hippocastanum*) in years 2011 and 2012

2011	*df*	Chlorophyll		Carotenoids		Anthocyanins		Phenolics		Flavonols		Carbohydrates		*df*	α-Aminoacids	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*		*F*	*p*
Individual	1	5.588	0.046	5.707	0.044	10.269	0.013	8.587	0.019	10.381	0.012	0.093	0.768	1	1.863	0.209
Sampling dates	4	4.618	0.005	3.769	0.013	38.254	0.000	73.223	0.000	14.953	0.000	48.443	0.000	3	55.043	0.000
Sampling dates × individual	4	8.073	0.000	6.714	0.000	3.120	0.028	0.654	0.629	1.208	0.326	0.176	0.949	3	9.731	0.000
2012	*df*	Chlorophyll		Carotenoids		Anthocyanins		Phenols		Flavonols		Carbohydrates		*df*	α-Aminoacids	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*		*F*	*p*
Individual	1	19.438	0.002	3.609	0.094	57.423	0.000	1354.134	0.000	6.461	0.035	77.244	0.000	1	4.995	0.056
Sampling dates	4	78.280	0.000	348.693	0.000	19.903	0.000	187.517	0.000	23.725	0.000	37.615	0.000	3	45.162	0.000
Sampling dates × individual	4	4.286	0.007	0.559	0.694	0.812	0.527	69.729	0.000	9.533	0.000	12.364	0.000	3	11.719	0.000

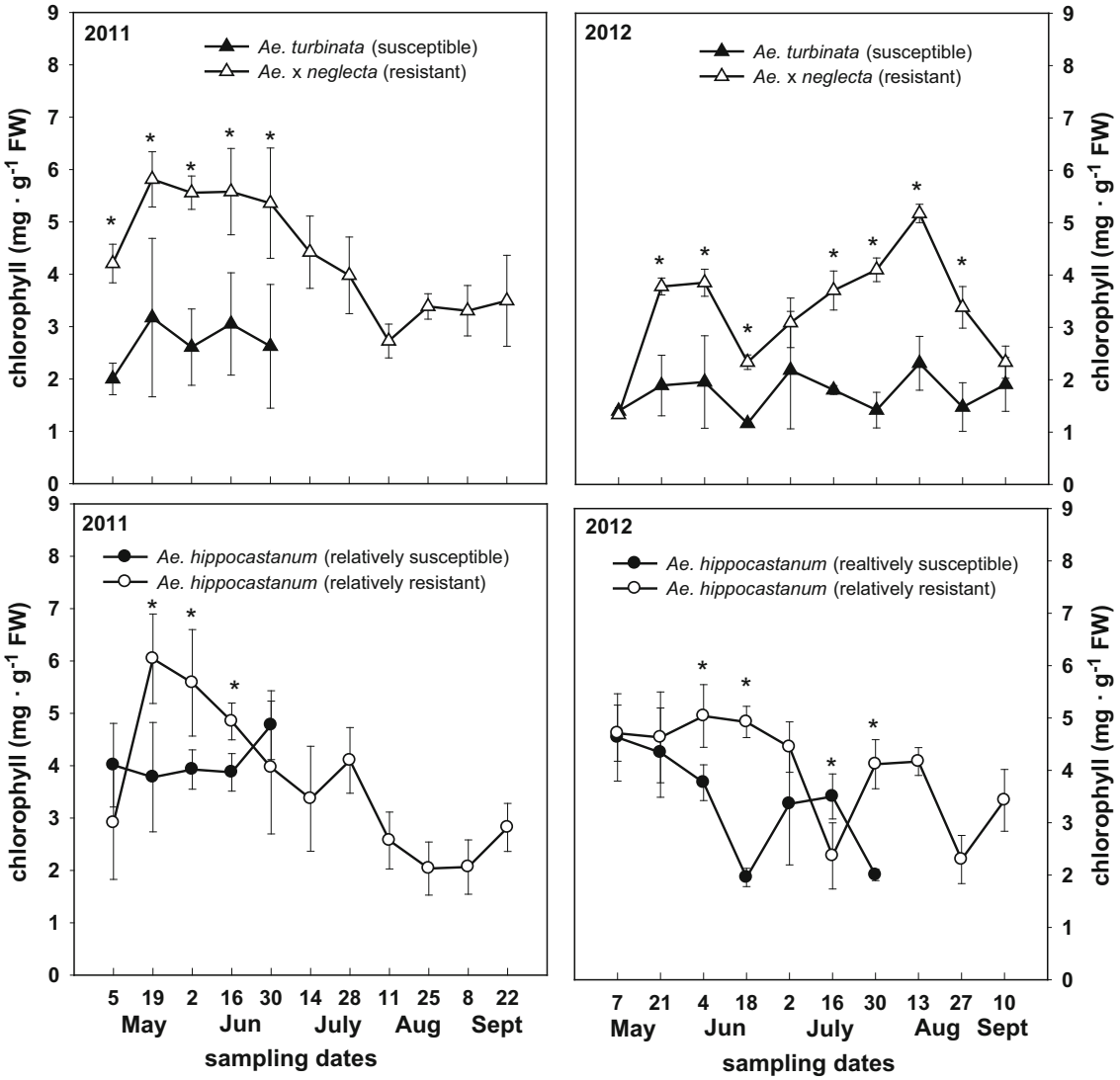

Fig. 2 Level of total chlorophyll in leaf blades of Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in years 2011 and 2012. Data are the means of five replications ± standard deviation marked as *vertical bars*. Significant differences between genotypes at the sampling dates are marked as *asterisk*

significantly higher than in susceptible *Ae. turbinata* (Table 2; Fig. 3). By contrast, although in 2012 the differences between these *Aesculus* trees in the level of carotenoid pigments were statistically significant (Table 2), they were lower than in 2011 (Fig. 3). The level of carotenoids in leaves of *Ae.* × *neglecta* was higher than *Ae. turbinata* but only on four sampling dates (21 May, 4 June, 30 July and 13 August). In the leaves of the resistant *Ae. hippocastanum* tree in 2011 a slightly higher carotenoid level was observed than in the relatively susceptible one. However, in 2012 there was no difference in the level of these compounds between examined white horse-chestnut trees (Fig. 3).

The pest-susceptible *Ae. turbinata* was characterized by a higher level of anthocyanins than resistant *Ae.* × *neglecta*, both in 2011 and 2012 (Fig. 4). A slight differences in the level of these pigments between the two *Ae. hippocastanum* trees differing in susceptibility to this pest were found in 2011 and 2012, but only on two or three dates (Fig. 4).

The content of phenolic compounds in 2011 and 2012 was significantly higher in the leaves of susceptible *Ae. turbinata* than resistant *Ae.* × *neglecta* (Fig. 5). Similarly, a higher level of these compounds was observed in the leaves of the pest-susceptible *Ae. hippocastanum* tree compared to the relatively resistant one (Fig. 5). Furthermore, the content of phenolic compounds in resistant *Ae.* × *neglecta* remained at a similar level at successive dates of determination. In *Ae. turbinata* and both specimens of *Ae. hippocastanum* their level increased in the course of feeding. It should be noted that the *Ae. hippocastanum* tree relatively resistant to horse-chestnut leaf miner was characterized by a twofold higher level of phenolic compounds in 2011 than in 2012. This

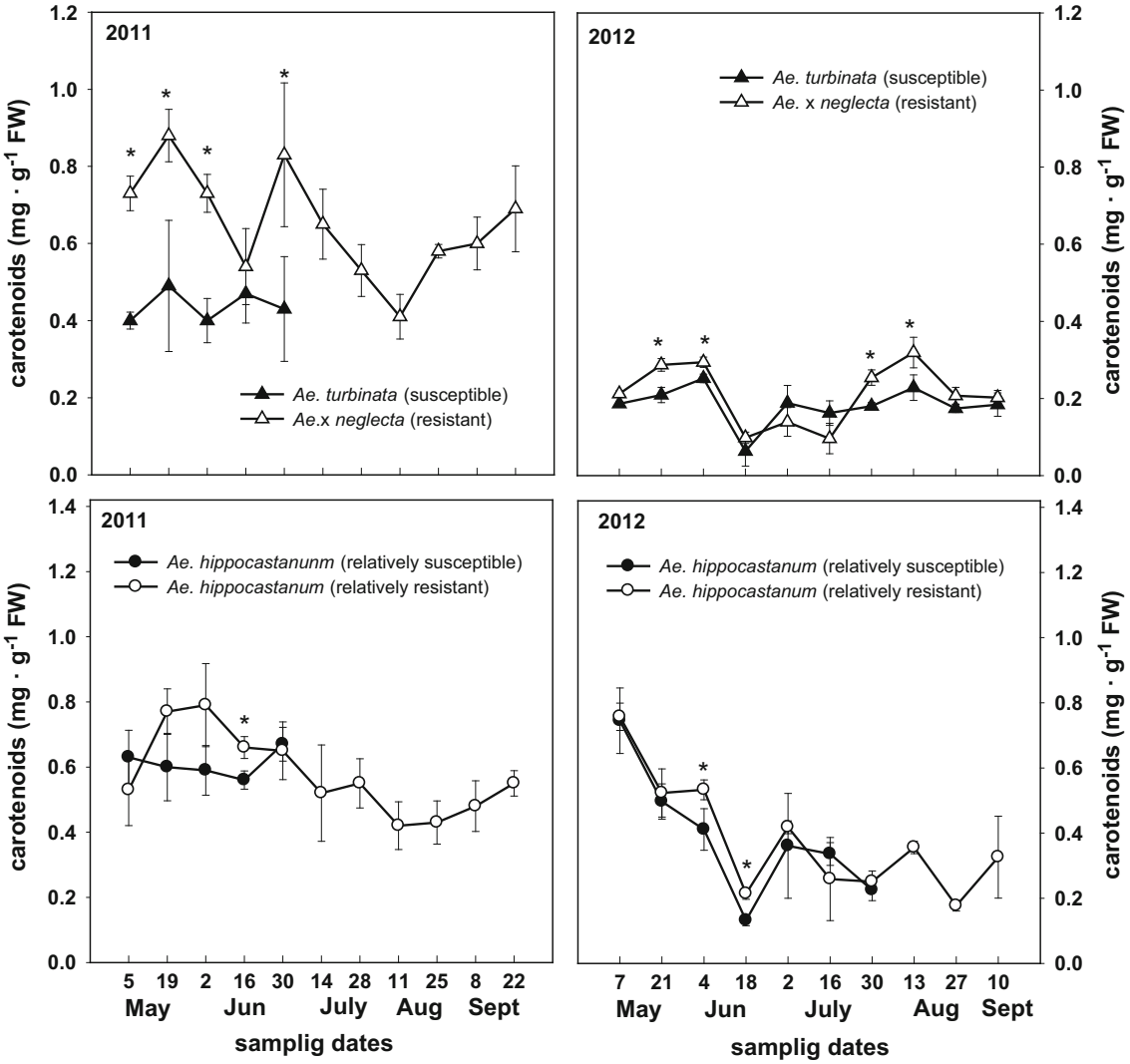

Fig. 3 Level of carotenoids in leaf blades of Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in 2011 and 2012. Data are the means of five replications ± standard deviation marked as *vertical bars*. Significant differences between genotypes at the sampling dates are marked as *asterisk*

could be associated with the more rapid development of the pest in 2012.

In 2011 a slightly higher level of flavonols but only on two dates was observed in the leaves of *Ae. turbinata* compared to *Ae.* × *neglecta*, whereas in 2012 these differences were larger, found on four dates (Fig. 6). Similarly, the pest-susceptible *Ae. hippocastanum* was characterized by a higher level of flavonols than the relatively resistant tree of this species and the observed differences were more pronounced in 2012 than in 2011 (Fig. 6).

In both years of experiments α-amino acid content was slightly higher in the resistant *Ae.* × *neglecta* than the susceptible *Ae. turbinata* (Fig. 7). There were no significant differences in the levels of these compounds between the two examined *Ae. hippocastanum* trees in 2011. However, in 2012 a slightly higher level of α-amino acids was observed in the relatively resistant *Ae. hippocastanum* tree (Fig. 7).

In 2011 as well as in 2012 the level of carbohydrates was significantly higher in susceptible *Ae. turbinata* than resistant *Ae.* × *neglecta* (Fig. 8). Leaf carbohydrate content in both *Ae. hippocastanum* trees was at the same level in 2011 but at most dates throughout 2012 was significantly higher in the relatively susceptible tree (Fig. 8).

Discussion

Weather conditions (air humidity, sunlight and temperature) are basic factors that influence phytophagous insects directly and indirectly (Jaworski and Hiszczanski 2013). Temperature exerts a significant effect on the development

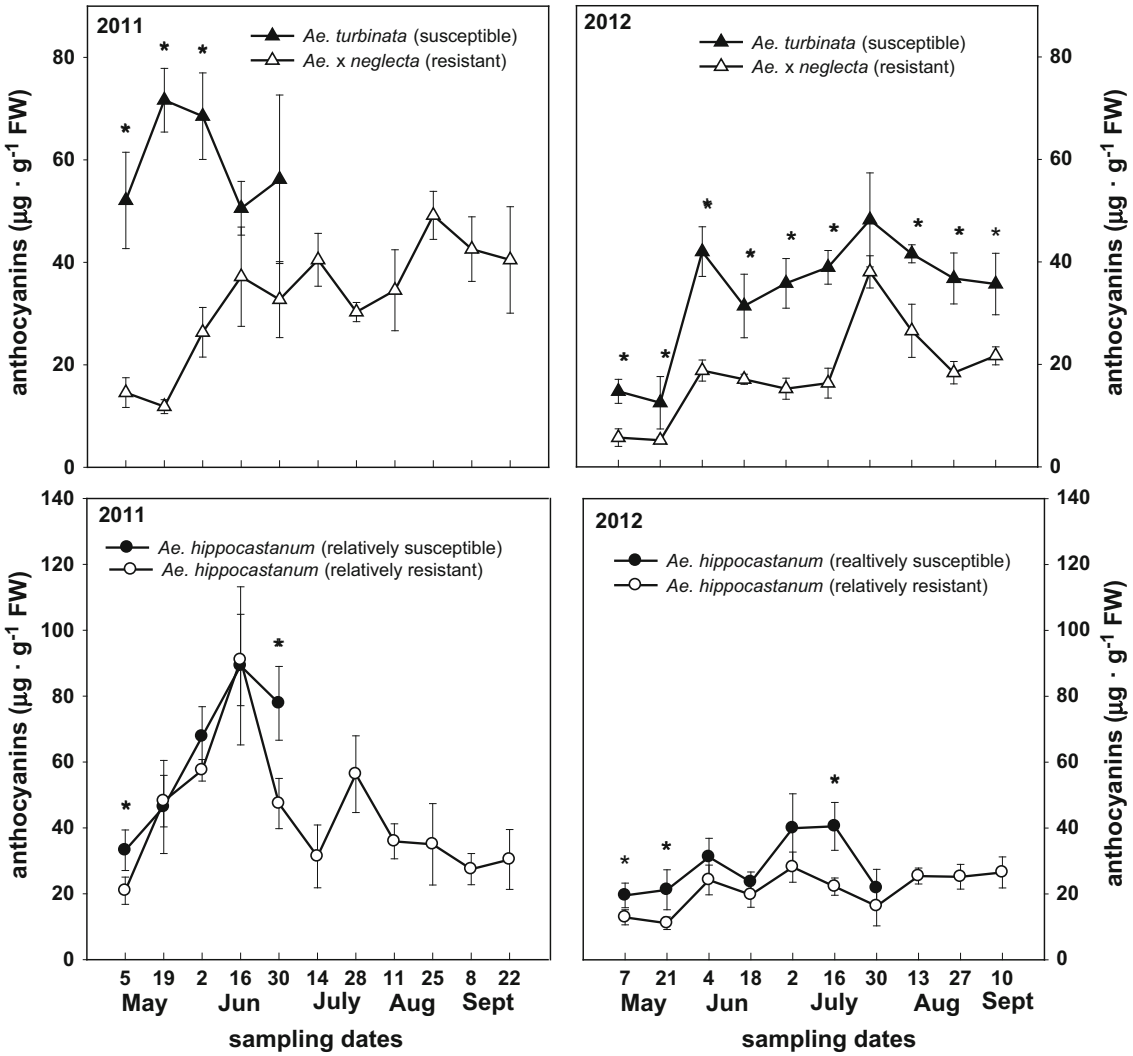

Fig. 4 Level of anthocyanins in leaf blades of Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in 2011 and 2012. Data are the means of five replications ± standard deviation marked as *vertical bars*. Significant differences between genotypes at the sampling dates are marked as *asterisk*

of *C. ohridella* (Dimić et al. 2000). Depending on climate conditions, the horse-chestnut leaf miner produces two to four generations per year. Usually in a warm dry summer *C. ohridella* is able to complete three generations (Tilbury et al. 2004; Girardoz et al. 2007). The flight period of the adults emerging from overwintering pupae (first generation) begins at the end of April and the beginning of May. The second generation usually appears in June-July and the third in August–September (Kukula-Mlynarczyk and Hurej 2007; Pál et al. 2014) The final generation overwinters in the stage of pupae, which are known to be extremely frost tolerant and survive in the fallen leaves (Tomiczek and Krehan 1998; Pál et al. 2014). It was reported that the density of the first and second generation of *C. ohridella* moths was greater during a warm than during a cool summer, which resulted in greater damage of horse-chestnut leaves (Kukuła-Młynarczyk and Hurej 2007). However, during a cool summer only two generations were developed and less damage of leaves was observed (Girardoz et al. 2007). Our research has shown that horse-chestnut individuals more susceptible to the pest were characterized by greater damage of leaves in 2011 than in 2012. This could be a result of slightly higher air temperature in April and June of 2011 than 2012, this being the period of larval development and emergence of the adults of the first and second generation.

A substantial influence on the behavior of insects is exerted by chemical composition of the host plants (Johne et al. 2006c; Johne et al. 2008; D'Costa et al. 2014). The reciprocal ratio of plant pigments contained in the leaf blades determines the final color of these organs and constitutes a visual signal which facilitates adult Lepidoptera to locate the host plant (Kočíková et al. 2012). The present results revealed that total chlorophyll content in the leaves

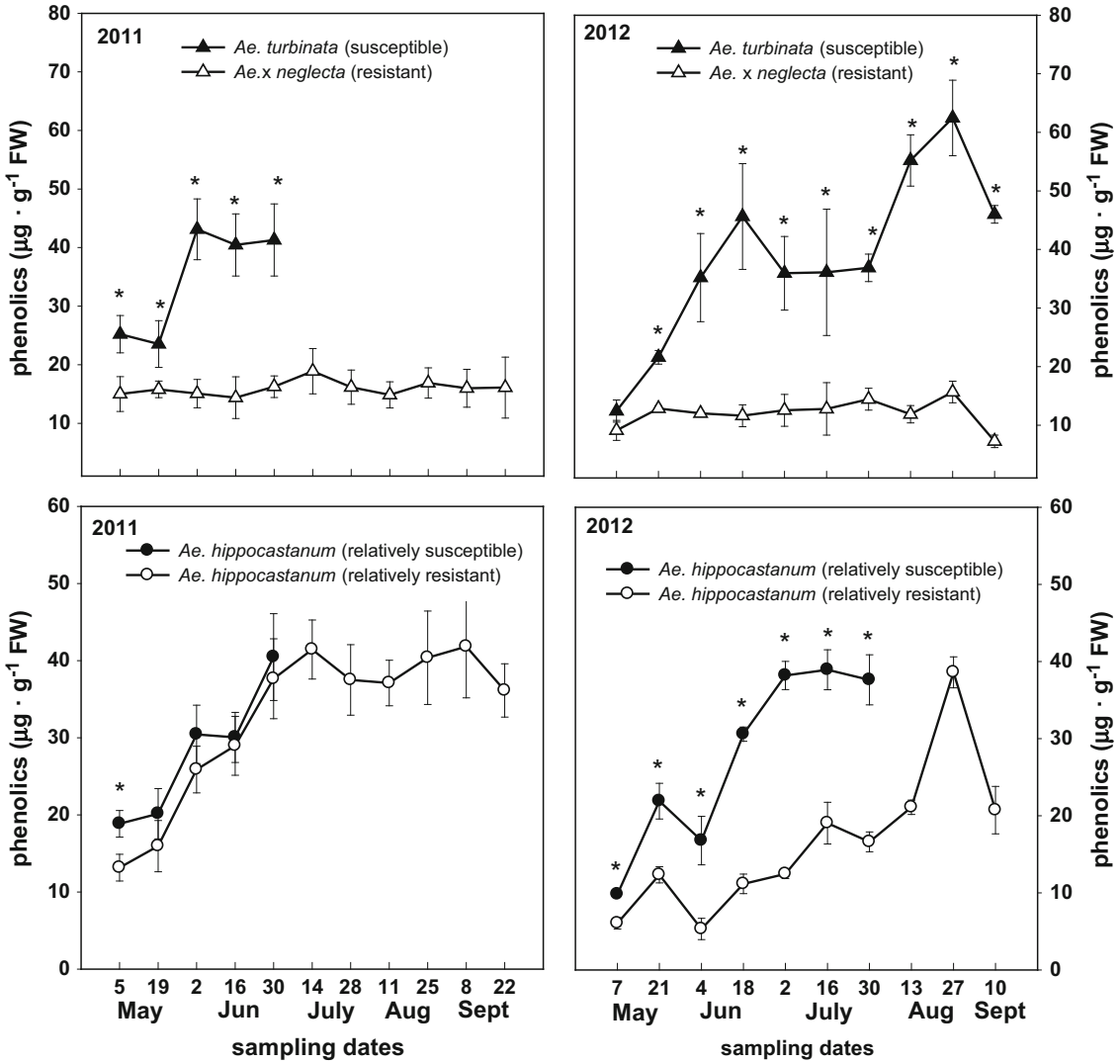

Fig. 5 Level of phenols in leaf blades of Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in 2011 and 2012. Data are the means of five replications ± standard deviation marked as *vertical bars*. Significant differences between genotypes at the sampling dates are marked as *asterisk*

of *Ae.* × *neglecta* completely resistant to *C. ohridella* was significantly higher than in *Ae. turbinata*, which is occupied by this pest. Similarly, higher leaf chlorophyll level in the relatively resistant tree of *Ae. hippocastanum* was coincident with the period in which it was less colonized by the horse-chestnut leaf miner than the susceptible one. This could mean that the intense green color of the leaves does not correspond to the visual pattern responsible for the location of host plants by the pest moths. On the other hand, Johne et al. (2006a) observed that the females of horse-chestnut leaf miner deposit more eggs on green leaf blades than on those which are covered by mines, because the oviposition and larval mining cause changes in the profile of leaf volatile compounds and odor pattern of leaves which inhibit other individuals from oviposition on the same leaves.

It should be noted that *Ae. turbinata* susceptible to *C. ohridella* was characterized by a higher level of anthocyanins in leaf blades than *Ae.* × *neglecta* on all of the dates in both growing seasons. The content of these pigments was higher at the beginning of the growing season before the appearance of the larvae. Therefore, it can be assumed that a higher level of these red pigments affects leaf coloration, recognized as a visual stimulus by females of horse-chestnut leaf miner looking for a place to deposit eggs. Similarly, the *Brassica rapa* genotype which was characterized by high concentrations of anthocyanins was more attacked by herbivorous insects than green leaf genotypes (Coelho 2004). On the other hand, there are numerous reports showing that higher levels of these pigments may inhibit infestation of leaves (Karageorgou and Manetas 2006; Karageorgou et al. 2008; Lev-Yadun and

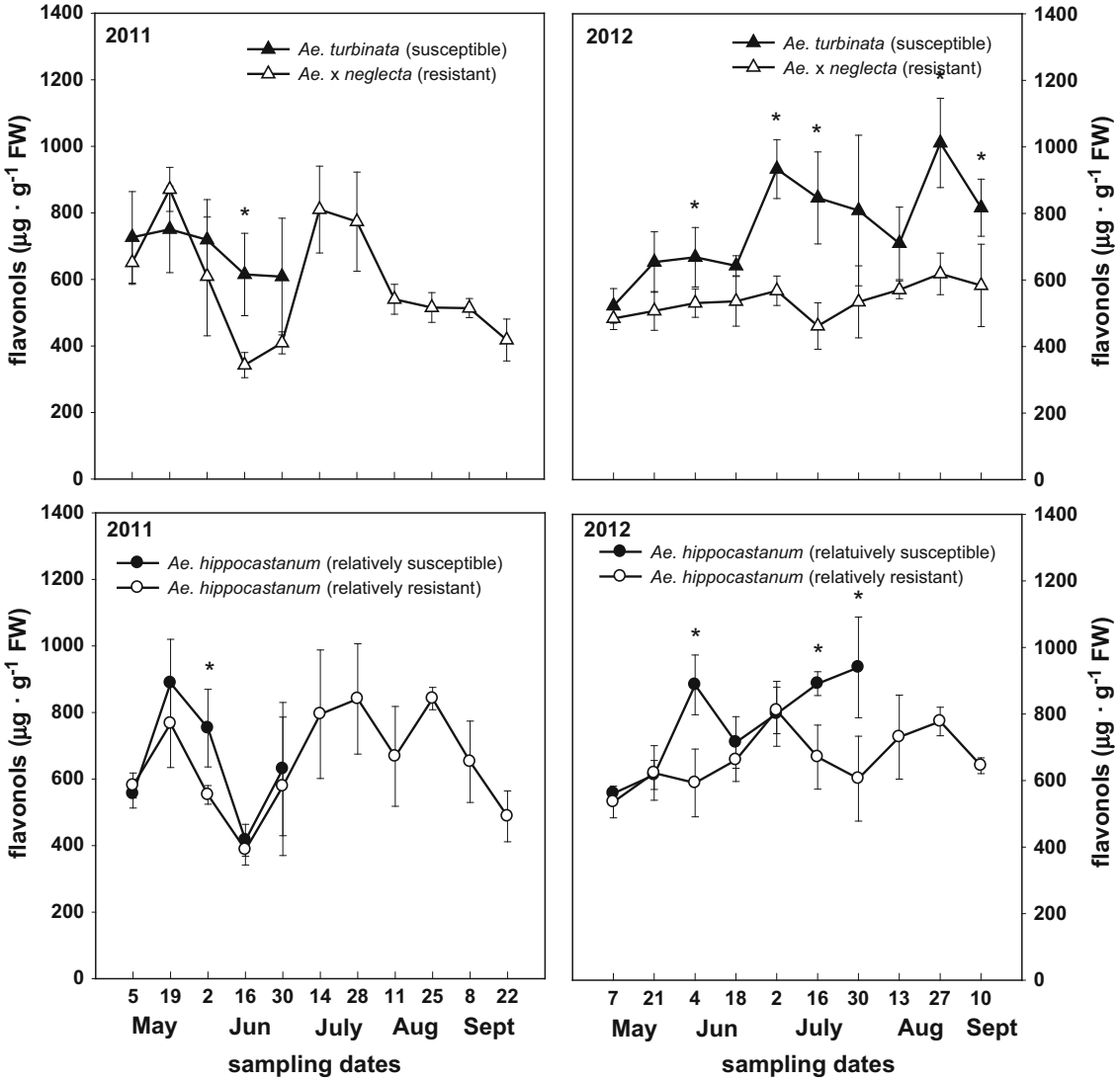

Fig. 6 Level of flavonols in leaf blades of Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in 2011 and 2012. Data are the means of five replications ± standard deviation marked as *vertical bars*. Significant differences between genotypes at the sampling dates are marked as *asterisk*

Gould 2009). Karageorgou and Manetas (2006) believe that insects avoid leaves rich in anthocyanins because they usually contain a lot of phenolics which act as defensive compounds. In fact, the results show that leaves of *Ae. turbinata* more abundant in anthocyanins contained significantly more phenolics than *Ae.* × *neglecta*, but these compounds did not affect the pests adversely because larvae developed well on this species. These results did not confirm the hypothesis that anthocyanins are compounds which may offer increased protection against phytophages. The results of some other studies also indicate that foliar anthocyanins are not toxic to insect herbivores and do not inhibit feeding (Schaefer and Rolshausen 2006).

Among the secondary metabolites, plant phenolic compounds represent a very important group of defensive compounds which play an essential role in resistance to insect herbivores (Ossipov et al. 2001; War et al. 2012). The enhancement of phenolic acids was observed in *Archis hypogaea* L. infested with three different Lepidoptera pests (Sambangi and Usha Rani 2013). A rapid induced defense response caused by an increased concentration of simple phenolics and monoterpenes was observed in silver birch infested by *Lymantria dispar* (Martemyanov et al. 2012). In our study the concentration of phenolics in the leaf blades of *Ae. turbinata* as well as in both individuals of *Ae. hippocastanum* increased with the development of the pest, which could be considered as a manifestation of chemical defense against feeding. It seems that these horse-chestnut trees expressly defended themselves but it was ineffective. It probably results from the fact that *C. ohridella* has an

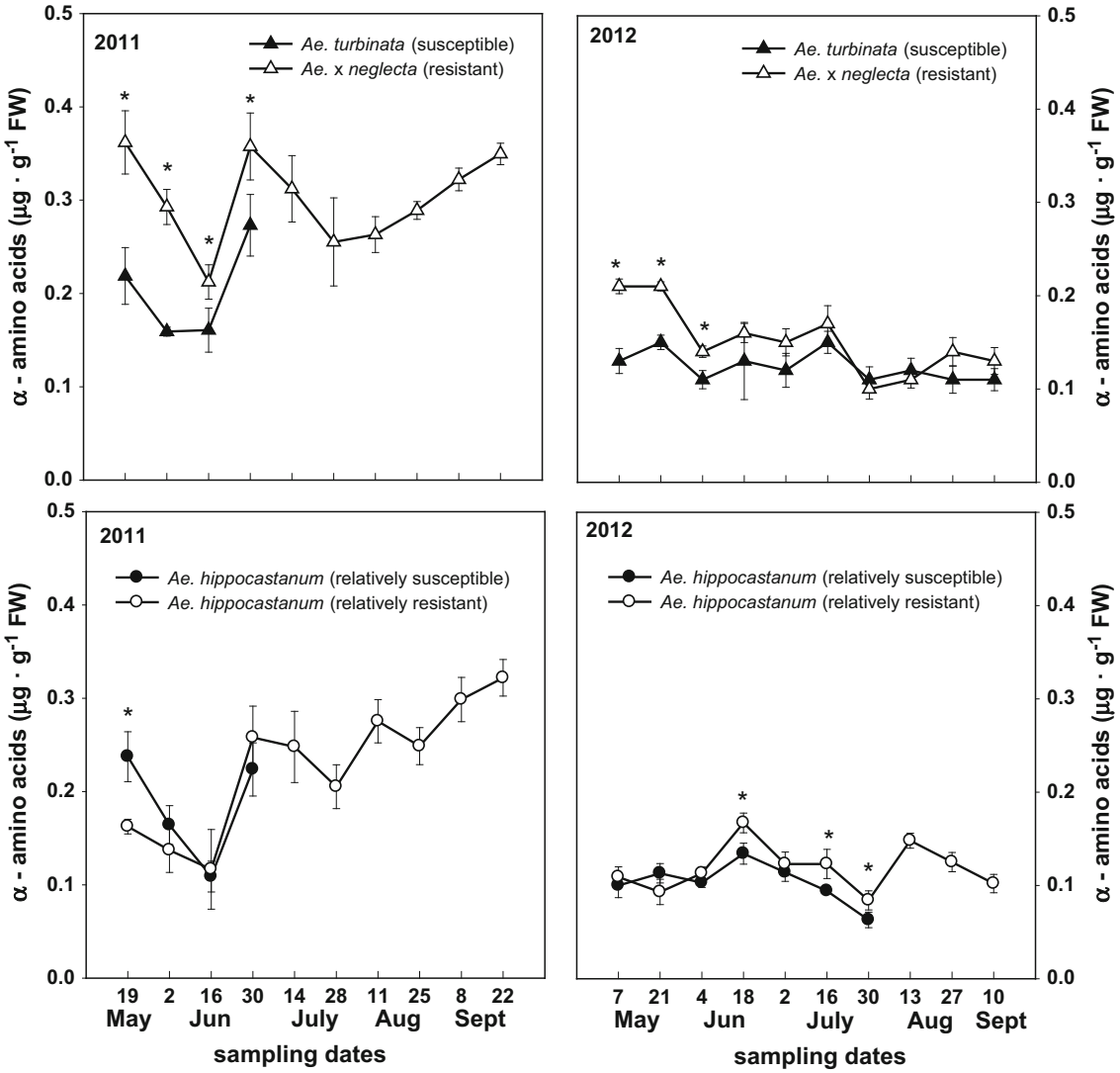

Fig. 7 Level of α-amino acids in leaf blades of Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in 2011 and 2012. Data are the means of five replications ± standard deviation marked as *vertical bars*. Significant differences between genotypes at the sampling dates are marked as *asterisk*

effective defense system which includes antioxidant enzymes and detoxifying enzymes, existing in the larvae midgut, responsible for detoxification of allelochemicals produced by host plants (Żaak et al. 2012). The highest activity of these enzymes was found in the midgut of the second larval generation which correlated with increased toxicity of food caused by the accumulation of secondary metabolites in leaves (Żaak et al. 2012). Our research also revealed that during both growing seasons the concentration of phenolics in leaf blades of susceptible *Ae. turbinata* was higher than in resistant *Ae.* × *neglecta*. These results do not show that phenolic compounds may be one of the factors responsible for resistance of the examined *Ae.* × *neglecta* individual to *C. ohridella*. Likewise, the results obtained by D'Costa et al. (2014) seem to contradict the role of leaf phenolics in resistance to the pest, because they showed that leaves of species susceptible (*Ae. hippocastanum, Ae. turbinata*) to *C. ohridella* were characterized by a higher level of phenolic compounds than leaves of resistant species (*Ae. chinensis*, *Ae. indica*) but also had a similar level of these compounds compared to the resistant *Ae. flava.* The results of other research revealed that red horse-chestnut resistant to *C. ohridella* was characterized by higher content of phenolic compounds, especially (−)-epicatechin and procyanidins, as well as a much higher level of saponins, compared to susceptible white horse-chestnut (Kukula-Mlynarczyk et al. 2006; Oszmiański et al. 2014). It may suggest that resistance to the pest depends on the qualitative rather than the quantitative profile of phenolic compounds.

Flavonols are compounds that have antinutritive activity and inhibit insect development (Adeyemi et al. 2010;

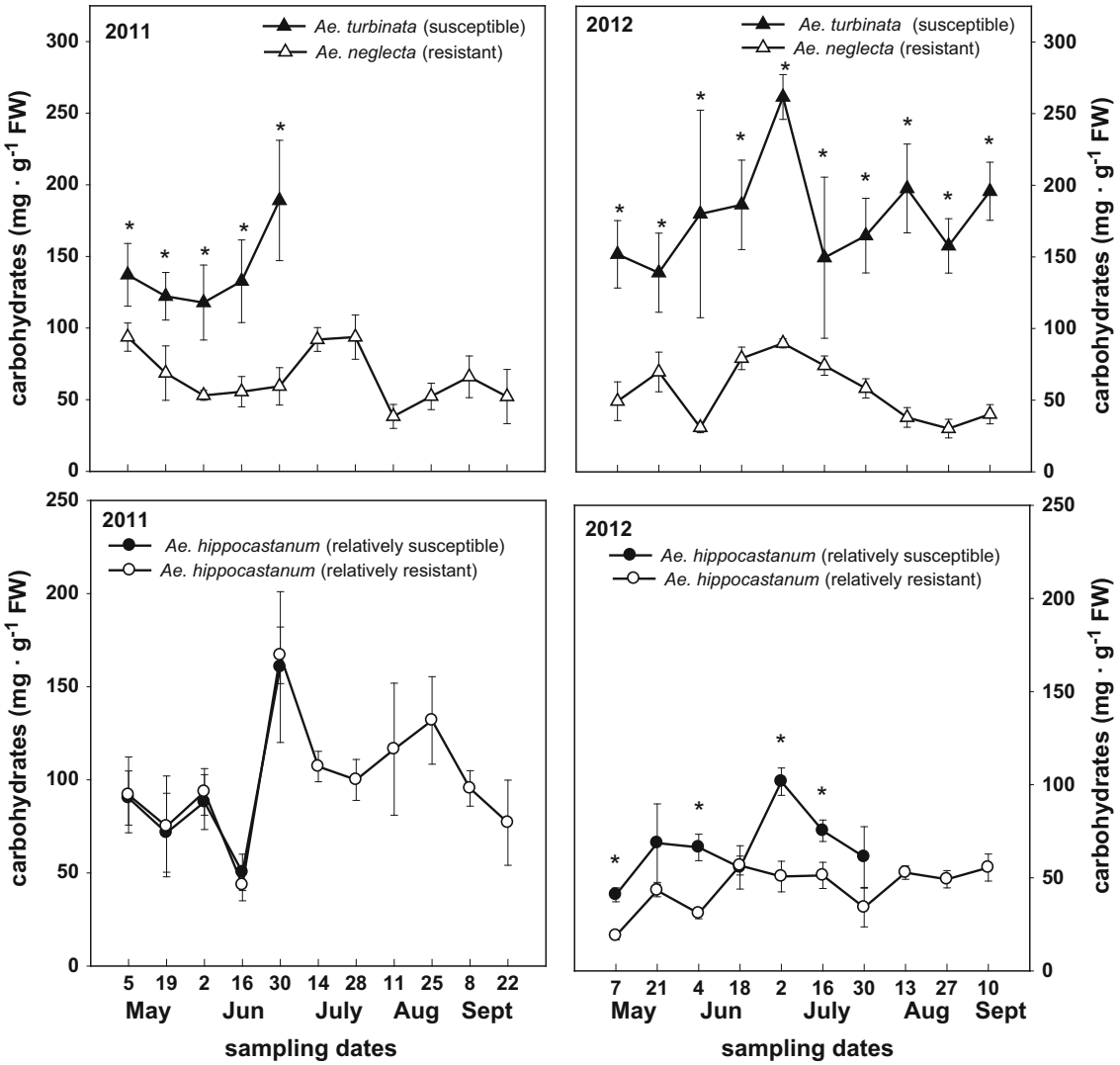

Fig. 8 Level of carbohydrates in leaf blades of Japanese horse-chestnut (*Aesculus turbinata*), painted buckeye (*Aesculus* × *neglecta*) and two trees of white horse-chestnut (*Aesculus hippocastanum*) in 2011 and 2012. Data are the means of five replications ± standard deviation marked as *vertical bars*. Significant differences between genotypes at the sampling dates are marked as *asterisk*

Martemyanov et al. 2012). A significant increase in the level of flavonols was observed in leaves of the *Ae. turbinata* tree in July 2012. After this date the damage of leaf blades did not increase. One can assume that the increase of flavonols could be caused by *C. ohridella* larval mining and resulted in the reduction of oviposition by conspecific females from the next pest generation. Therefore, the inhibition of further damage may be caused by induced chemical defense in which flavonols play an important role (War et al. 2012).

An important factor which stimulates larvae to feed is the abundance of nutrients in leaves (Bede et al. 2007). Insects more willingly select leaves that are rich in amino acids and carbohydrates (Glendinning et al. 2007). The level of amino acids in the leaves may be an important factor affecting the size of the phytophagous population. Insects that feed on tissues rich in amino acids grow faster, are larger and more fertile, and their progeny have a greater chance of survival in comparison to insects which feed on food containing a low level of these compounds (Cahenzli and Erhardt 2013; Marchioro and Foerster 2013). Our results do not confirm that the level of amino acids is a factor which affects the feeding of the horse-chestnut leaf miner. In both years throughout May and June the pest-susceptible *Ae. turbinata* had a lower amino acid content in leaf blades than the resistant *Ae.* × *neglecta*, whereas in both specimens of *Ae. hippocastanum* the content of amino acids was at a similar level throughout both growing seasons. Similarly, in other studies amino acid level in leaves of *Aesculus* species was not a trait which favored oviposition preference of *C. ohridella* and larval feeding (D'Costa et al. 2014). In the midgut of *C. ohridella* there

are many enzymes catalyzing carbohydrate degradation, which indicates that these compounds are important nutrients for this pest (Stygar et al. 2010). The results of our study confirm the important role of carbohydrates in the stimulation of feeding of the horse-chestnut leaf miner. The pest-susceptible *Ae. turbinata* tree was found to have, in both growing seasons, a significantly higher level of leaf carbohydrates than the resistant *Ae.* × *neglecta*, while the relatively susceptible *Ae. hippocastanum* tree contained a slightly higher level of carbohydrates than the relatively resistant ones, but only in 2012. Slightly different and ambiguous results were obtained by D'Costa et al. (2014), who found that *Ae. hippocastanum* and *Ae. turbinata* susceptible to *C. ohridella* had similar carbohydrate contents compared to the resistant *Ae. chinensis* and *Ae. indica* species, and all these species had lower carbohydrate levels than *Ae. flava*, resistant to the pest.

In conclusion, the obtained results revealed that traits which may influence oviposition preference and favor the feeding of *C. ohridella* include anthocyanin and carbohydrate levels. The higher leaf carbohydrate content in the susceptible *Ae. turbinata* tree compared to resistant *Ae.* × *neglecta* suggests that the leaves of the former tree are more valuable food resources for larvae of this pest. Moreover, it may be speculated that the higher anthocyanin level in leaves of the *Ae. turbinata* specimen affects the leaf color, which may be an attractive visual stimulus for females of *C. ohridella*. However, the differences in the level of these compounds between the two *Ae. hippocastanum* trees, although statistically significant, are slight and do not explain their different susceptibility. The current results demonstrate that the examined leaf traits do not constitute a complete description of relationships between *C. ohridella* and examined trees of the genus *Aesculus*. The chemical composition of horse-chestnut leaves is probably more complex, and many other biochemical and physiological traits are responsible for the multifaceted interaction between this pest and different genotypes of *Aesculus* sp. The important results of Irzykowska et al. (2013) showed significant inter-species genetic variation within an *Ae. hippocastanum* population grown in urban green spaces, and 17 molecular markers strongly associated with their susceptibility to *C. ohridella* were found. Broader and more extensive investigations are needed in order to determine the traits that make some *Aesculus* trees more attractive and why they are willingly colonized by this pest while others are not colonized.

Author contribution statement MP—experimental design, sample collection and chemical analysis in 2011 and 2012, preparation of the manuscript; HB—data analysis, discussion of results and preparation of the manuscript; JW—chemical analysis in 2012; KM and MG—statistical analysis and interpretation of results.

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